Epidemiologic typing of international collections of *Klebsiella* spp.: computerized biochemical fingerprinting compared with serotyping, phage typing and pulsed-field gel electrophoresis

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Objective: The Phene Plate (PhP) biochemical fingerprinting system is based on measurements of the kinetics of selected biochemical reactions performed in microtiter plates, and computerized data-processing. This study compared the performance of the PhP system as an epidemiologic tool with other commonly used typing systems.

Methods: PhP typing was applied to 107 nosocomial *Klebsiella* spp. isolates from 10 collections, mostly representing outbreaks. The results were compared with those obtained by capsular (K) serotyping, phage typing and, for a subset of isolates (n=33), pulsed-field gel electrophoresis (PFGE).

Results: Clusters of identical or closely related isolates based on serotype, phage type, PhP type and PFGE type were found in most collections. The typeability was 100%, 95%, 94% and 68% for PhP, K, PFGE and phage typing, respectively. The agreement between the typing methods was high (88–96%). The discriminatory power was high for PhP and PFGE (diversity index 0.95 and 0.97, respectively), but lower for phage typing (diversity index 0.91) and K typing (diversity index 0.87).

Conclusions: Like serotyping and PFGE, PhP typing is useful in studies of the nosocomial epidemiology of *Klebsiella* spp. Combining PhP typing with PFGE or K typing rarely yielded additional information when comparing isolates within each collection, but PFGE sometimes discriminated between isolates of similar PhP type derived from different collections.

Key words: epidemiologic typing, *Klebsiella* spp., biochemical fingerprinting, K typing, pulsed-field gel electrophoresis

INTRODUCTION

The genus *Klebsiella* is widely distributed in nature and may also be a resident in the human bowel. Occasionally, these organisms invade the urinary tract, wounds and bloodstream and cause serious infections [1]. *Klebsiella* spp. are usually second to *Escherichia coli* among Gram-negative organisms causing hospital-aquired infections, and are often involved in nosocomial outbreaks [2–4].

Klebsiella spp. can be subdivided into 77 capsular (K) serotypes, but a comprehensive panel of antisera must be maintained for serotyping. Approximately six serotypes account for >40% of clinical isolates [2]. In investigations of cross-infection and outbreaks, phage typing can be used for subdivision of frequent serotypes [5]. Nevertheless, the combined discriminatory power of these two typing systems may be inadequate for the investigation of complex outbreaks, and additional or alternative methods offering improved discrimination, such as pulsed-field gel electrophoresis (PFGE), plasmid profiling, random amplified polymorphic DNA typing, or ribotyping, are therefore sometimes used [2,6–9].

Traditional phenotyping systems are not regarded as being sufficiently discriminatory and stable to be useful in epidemiologic work. We have developed a highly reproducible and discriminatory typing method based on measurements of the kinetics of biochemical reactions and computerized data-processing (the Phene Plate or PhP system) [10]. The PhP system has the advantage of being relatively inexpensive and easy to perform, while allowing comparison of large numbers

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of bacterial isolates. The system consists of microtiter plates containing different sets of biochemical reagents and has been used in various epidemiologic studies [11–16].

The PhP system adapted for typing of *Klebsiella* spp. showed high discriminatory power when used on epidemiologically unrelated isolates [10], and has previously been used alone, or together with serotyping, to study the transmission of fecal *Klebsiella* strains among infants in special care neonatal units [14,16,17]. In the present evaluation of PhP typing, this system was compared with K serotyping, phage typing and PFGE of total bacterial DNA in an investigation of collections of *Klebsiella* spp. from defined epidemiologic situations in different countries.

MATERIALS AND METHODS

Bacterial isolates

One hundred and seven isolates of *Klebsiella* spp. (*K. pneumoniae*, $n=74$, and *K. oxytoca*, $n=33$) from 10 European and Australian collections were studied (Table 1). Most isolates were submitted for typing either because of a suspected hospital outbreak or for screening of aminoglycoside resistance. One collection (no. 4) was from a transmission (hand transfer) study. Collection 10 consisted of isolates from two separate outbreaks in the same intensive care unit (ICU).

PhP typing

A semi-automated system for biochemical fingerprinting (the PhenePlate or PhP system, BioSys inova, Stockholm, Sweden) was used to identify biochemical phenotypes (PhP types). The system consists of 96–well microtiter plates, each containing two sets of 48 different dehydrated reagents selected to yield a high degree of discrimination among isolates belonging to various Enterobacteriaceae, and a specially designed computer software program for data-processing. For typing of *Klebsiella* spp. and other Gram-negative bacteria, 32 of the 48 reactions were shown to be the most useful [10,13], and only those reactions were used for data evaluation in the present study.

A colony of each isolate to be tested was suspended in 12 mL peptone broth, containing bromothymol blue as a pH indicator, and 0.15 mL of the suspension was dispensed into each of the wells in the PhP system. The plates were incubated at 37°C, and the absorbance of each well was recorded at 7 h, 24 h and 48 h by a microtiter plate reader connected to a computer. For each of the 32 most useful reactions, the mean value of all readings was calculated, yielding biochemical fingerprints consisting of a set of 32 numbers for each isolate. The fingerprints were compared pairwise and the correlation coefficient (r) between each pair of isolates was calculated. Isolates with correlation coefficients equal to or higher than the identity level ($r=0.97$, defined by the reproducibility of the method) [12], were regarded as identical and assigned to the same common PhP type (C phenotype), while isolates with lower correlation coefficients when compared with all other isolates were defined as single (S) types. As this study indicated that local isolates of *Klebsiella* spp. with r values of 0.95–0.97 usually belonged to the same cluster according to K typing and PFGE, such isolates were designated variants of a C type defined by PhP (e.g. C1a, C1b). For each set of compared isolates, a similarity matrix was obtained and clustered by the UPGMA method so that the results could be presented as a dendrogram [18].

In order to study the relatedness of PhP types within serotypes, isolates belonging to K types found in more than one of the 10 collections were subjected to cluster analysis. The biochemical homogeneity within each of these serotypes was calculated as the mean correlation coefficient (mean r) obtained from pairwise comparisons of all PhP types identified within the serotype.

Table 1 Origin and major K serotypes of the isolates of *Klebsiella* spp. in the collections studied

Collection no.	No. of isolates	Country of origin	Method of selection	Major K types found[a]
1	7	England	Routine screening	2
2	12	Australia	Referred outbreak	54, 1
3	8	England	Referred outbreak	8
4	7	England	Transfer study	21
5	13	France	HIV patients	62, 2, 8
6	13	Australia	Referred outbreak	8, 54
7	7	England	Routine screening	21
8	15	England	Referred outbreak	21
9	6	England	Routine screening	39, 3
10	19	Belgium	Referred outbreaks[b]	28, 54, 62
Total	107			

[a]Types represented by two or more isolates within a collection.
[b]Two sets of isolates from the same intensive care unit.

K serotyping

Serotyping based on capsular polysaccharides was performed by countercurrent immunoelectrophoresis with polyclonal antisera for each of the 77 K types of *Klebsiella* spp. [19].

Phage typing

Phage typing was performed as described by Gaston et al [5].

PFGE

Representative isolates from clusters of PhP and/or K types, as well as isolates with discrepant serotyping and PhP typing results, were further characterized by PFGE of total bacterial DNA digested with restriction endonuclease *Xba*I (Promega, Madison, WI, USA) [7]. Preparation and digestion of DNA was in plugs of InCert agarose (FMC BioProducts, Rockland, Maine, USA), following the manufacturer's protocol for *E. coli* cells, except that buffer volumes were decreased and incubation times were shortened. Restriction fragments were resolved in FastLane agarose (FMC BioProducts) 1% w/v and 0.5× TBE buffer [20] at 200 V for 16 h, with pulse times increased linearly from 30 to 75 s, with a Gene Navigator system (Pharmacia Biotech, Uppsala, Sweden). The PFGE patterns were visually assessed. Isolates with indistinguishable patterns were considered to belong to the same PFGE type and were designated by the same unique number (e.g. 1). Isolates with patterns closely related to that of an already defined type were designated by the number of that type followed by a letter (e.g. 1a, 1b).

Comparison of typing methods

The discriminatory power of each typing system was defined as the diversity among all typeable isolates studied by Simpson's diversity index [21]. The agreement between the typing systems was calculated by comparing all typeable isolates pairwise, and by calculating the proportion of pairs that were either identical or different by the typing methods examined [15].

RESULTS

Overall performance of the typing methods

Thirty-four PhP types, 16 K types and 17 phage types were identified among the 107 isolates of *Klebsiella* spp. in the 10 collections studied. The typeability was 100% for PhP typing, 95% for serotyping and 68% for phage typing, while 31 of 33 isolates studied by PFGE could be assigned to distinct types. The discriminatory power (diversity index) was higher for PhP and PFGE than for K serotyping and phage typing (Table 2). The pairwise agreements between the different methods were: 88% for K and phage typing; 91% for K and PhP typing; 93% for PhP and phage typing; 93% for PFGE and K typing; 94% for PFGE and phage typing; and 96% for PFGE and PhP typing.

Analysis of the collections

Cluster analysis results of the PhP results for isolates from each collection were compared with the serotypes, phage types and, when available, PFGE types.

Table 2 Performance of methods used for typing of 107 *Klebsiella* spp. isolates[a]

Method	Typability (%)	No. of types	Diversity index[b]
K typing	95	16	0.87
Phage typing	68	17	0.88
PhP typing	100	34	0.94
PFGE[a]	94	15	0.96

[a]PFGE typing was applied to a subset of isolates (n=33).
[b]Simpson's diversity index.

One or two clusters of isolates of identical K type and identical or similar PhP type and PFGE type were found in all collections (results not shown). Collections 4, 6 and 8 consisted mainly of identical isolates irrespective of the typing method, whereas in collections 2 and 3, all isolates of the dominant cluster had the same K type, but slightly varying PFGE and PhP types. Collection 10, which represented two outbreaks in the same ICU, contained three dominant K and PFGE types, two of which were of similar PhP type.

Analysis of the K types

Six K serotypes were represented by isolates from more than one collection. When their PhP types were subjected to separate cluster analysis, all K54 isolates were of closely related PhP type (mean r 0.94), irrespective of origin. In contrast, the other widespread K types were biochemically diverse (mean r 0.56–0.81).

Correlation between the typing methods

All typing methods correlated well for collections 4, 6 and 8, while collection 5 had a dominant strain of homogeneous K, PhP and PFGE type, but variable phage type. Collections 1, 2 and 3 contained clusters of isolates, each with their own K types and identical or similar PhP and PFGE type, but these isolates were non-typeable by phage typing. The isolates in collection 1 were also non-typeable by PFGE. Among the K21 isolates, phage and PFGE typing discriminated between isolates of similar PhP types that originated from two different UK collections.

The results of all four typing methods were compared for 33 isolates from nine collections (Figure 1). There was good agreement between the methods for most isolates. Typically, isolates belonging to a cluster had a stable K type and little or no variation in PFGE and PhP types, but were sometimes non-typeable with phages. One cluster (from collection 4) was unusual in that the PhP types of the isolates were similar, despite the fact that they comprised two distinct strains according to K, PFGE and phage typing (Figure 1).

DISCUSSION

The PhP system is a commercially available typing method based on biochemical reactions and numerical data analysis. In contrast to conventional biochemical tube tests and commercially available kits intended for species identification (e.g. API or Biolog), PhP uses reagents selected to be discriminatory below the species

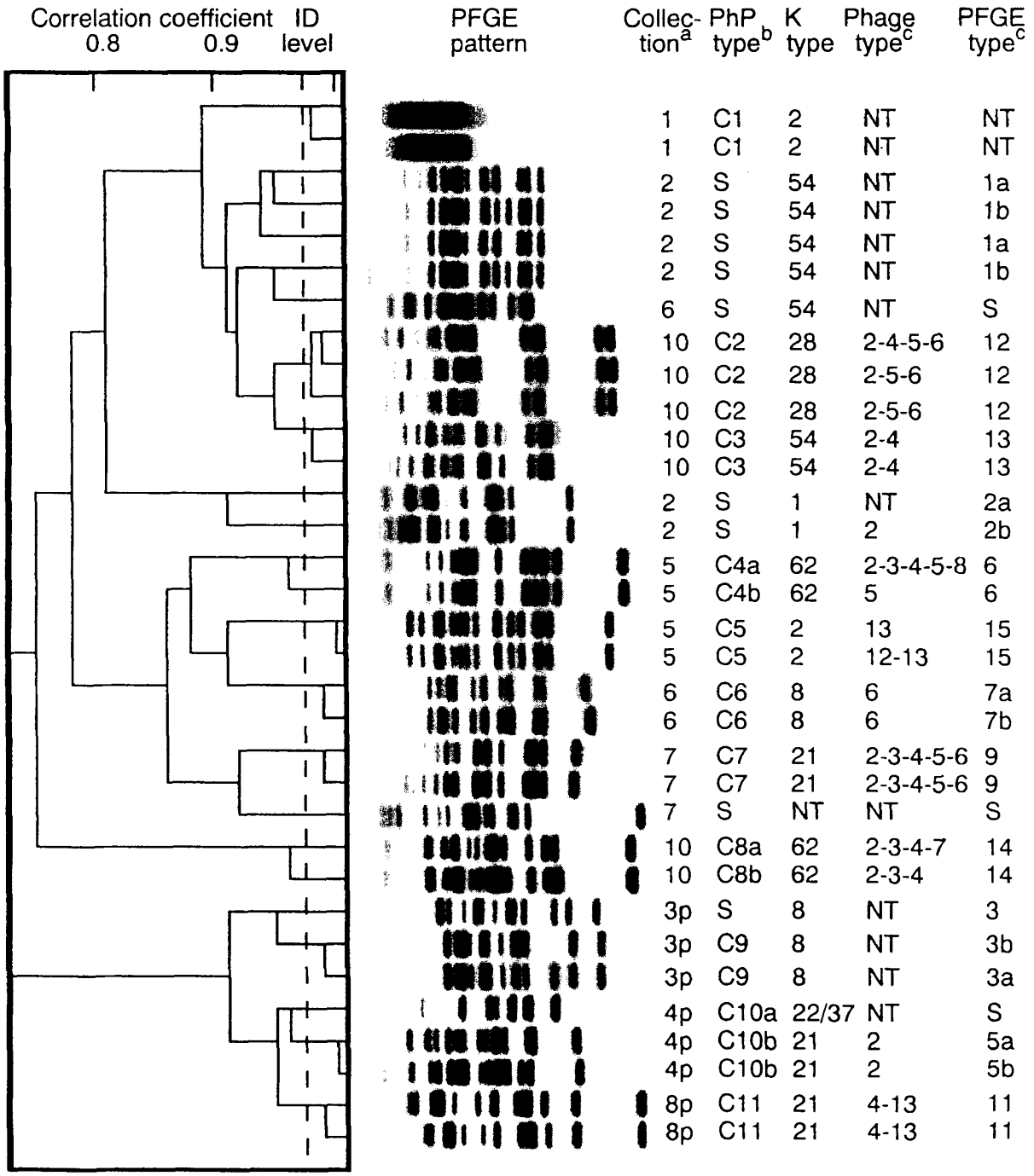

Figure 1 Cluster analysis of PhP typing results for the 33 isolates that were subjected to PFGE analysis, with K types, phage types and PFGE types added. The PFGE patterns were derived from different gels which were normalized and are shown here as a reconstructed gel image created by Gelcompar software (Applied Maths, Kortrijk, Belgium).
[a]p, *K. pneumoniae* (all other isolates are *K. oxytoca*); [b]S, single PhP type; [c]NT, non-typeable

level. Furthermore, PhP typing is based on the kinetics of each reaction instead of only a plus or minus value. This yields a high discriminatory power and, because variable interpretations of intermediate reactions are eliminated, also a high level of reproducibility (>95%) [10]. Compared to modern molecular typing methods, which require preparation of bacterial proteins, DNA, or other cell components, combined with gel electrophoresis and sometimes repeat runs of isolates with similar patterns on the same gel, PhP typing is rapid (up to 500 isolates may be run and analyzed by one person during one working week). It does not require technical expertise or expensive equipment other than a microplate (ELISA) reader and a computer with PhP software. The computerized data-processing allows the comparison of an almost unlimited number of isolates tested on different occasions.

The definition of identity between isolates in the PhP–KE system was set at a correlation coefficient (r value) of ≥ 0.97, since independent duplicate assays of the same isolate yielded correlation coefficients equal to or above this level in 95% of cases [10]. It has been shown that the r values within strains of enterobacteria may decrease after a series of subcultivations because of a drift of their biochemical activities in vitro [22]. In the present study, some isolates of *Klebsiella* spp. with a common origin had r values slightly lower than 0.97, indicating that an outbreak strain may evolve and diverge over time. A similar phenomenon was also observed for some of the PFGE patterns in which isolates differed by only one band. Thus, in contrast to serotyping, both PFGE and, particularly, PhP typing allow grading of the relatedness of isolates, thereby adding a new dimension to typing.

In the present study of outbreaks, the formal performance of PFGE and PhP typing was superior to that of K typing and phage typing with regard to typeability and discriminatory power, thus minimizing the number of non-typeable isolates and chance identity between isolates. Nevertheless, K typing was an equally good epidemiologic tool, as it correctly grouped most isolates that were thought to have a common origin. The facilities, technical skill and time required for these methods are, however, markedly different. Appropriate sera for serotyping are rarely available and, as stated by Tenover et al in a recent review, PFGE appears to be best suited for examination of relatively small sets of isolates ($n \leq 30$) [8], whereas these limitations do not apply to PhP typing.

It was concluded that both PhP and PFGE typing are useful methods for analyzing nosocomial isolates of *Klebsiella* spp. PhP typing has the advantage of allowing easy typing of large numbers of isolates and may therefore be especially useful as a first screening method. PFGE typing or serotyping could be used to verify results for isolates where PhP typing is not discriminatory enough or where stronger epidemiologic evidence is required.

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